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Recombinant Vaccinia Viruses Carrying the N Gene of Human Respiratory Syncytial Virus: Studies of Gene Expression in Cell Culture and Immune Response in Mice

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The construction and characterization of vaccinia virus recombinants carrying the nucleocapsid (N) protein gene of human respiratory syncytial (RS) virus are described. Recombinant viruses were constructed that contained the N gene oriented either positively or negatively with respect to the 7.5-kilodalton vaccinia virus promoter. In addition, a positively oriented recombinant was constructed that lacked an out-of-frame AUG codon in the 5′-terminal noncoding region. In HEp-2 cells, both positive-orientation recombinants induced the synthesis of a protein which comigrated with N protein and was precipitated by antisera to RS virus. Sera from mice immunized with these recombinants specifically precipitated the RS virus N protein. Analysis of mRNA and protein expressed from the recombinant N genes showed that deletion of the upstream AUG codon markedly improved the efficiency of protein synthesis. Mice were vaccinated with the high-expressing recombinant and subsequently challenged with live RS virus. The results of these experiments demonstrated that the immune response to N protein afforded a significant degree of protection against RS virus disease.

Human respiratory syncytial (RS) virus, a paramyxovirus of the pneumovirus taxon, is a leading cause of severe lower respiratory tract infection in children. No safe and effective vaccine is currently available for human use. Experimental vaccination of children with inactivated RS virus actually increased the severity of the disease (11). This, and the fact that individuals may become reinfected with antigenically related strains of RS virus at intervals of 2 to 3 years (9), highlights the problem of stimulating and maintaining immunity in the human host.

In an effort to dissect the role of individual RS virus proteins in the pathogenesis of RS virus disease and to analyze the various components of the immune response to RS infection, vaccinia virus recombinant vectors have been used to express individual RS virus genes in cell culture and in experimental animals. Vaccinia virus recombinants carrying DNA copies of the genes encoding the glycoprotein (G) and fusion glycoprotein (F) of human RS virus have been described (1, 6, 16, 26). Infection of mice (20, 26) or cotton rats (6, 16) with recombinant viruses expressing either protein elicited a strong humoral antibody response and conferred significant protection against live-virus challenge. However, studies with immunodeficient hosts show that a functional cellular immune system assists in clearing RS virus infections (7). It may also be significant that the vaccine recently developed for use against bovine RS virus is made from virus-infected bovine cells (22), rather than free virus, and its potency could well be due to an ability to induce some kind of cell-mediated immune response. Interestingly, the internal nucleocapsid (N) proteins of both influenza (25, 30) and vesicular stomatitis (17) viruses are major target antigens for cytotoxic T lymphocytes. It was of interest, therefore, to determine whether the equivalent nucleocapsid protein of RS virus would also be able to stimulate a protective immune response. This paper describes (i) the construction of vaccinia virus recombinants that carry the N protein gene of human RS virus, (ii) an analysis of the RS virus-specific transcription and translation products of these recombinant vectors in infected cells, and (iii) a study of their efficacy as live-virus vaccines in mice. In conjunction with this work, recent experiments with these recombinants have shown that the N protein is recognized as a target antigen by human and murine RS virus-primed cytotoxic T lymphocytes (2).

MATERIALS AND METHODS

Vaccinia virus insertion plasmids. A cDNA copy of the complete N gene of RS virus was made by fusing clones N257 and N6-63 (4) at the *Xba*I site (D. Malinowski and R. Pearson, unpublished data). A 1.6-kilobase (kb) *Aha*III fragment from this construct, containing the N gene, was cloned into the *Sma*I site of a recombinant plasmid that contained the vaccinia virus 7.5-kilodalton (kDa) promoter in the *Eco*RI site of the thymidine kinase (TK) gene (20) to produce vector pSL125 shown in Fig. 1A. Insertion of the same DNA fragment in the opposite orientation generated insertion plasmid pSL73 (not shown). Plasmid pAQ330 was derived from pSL125 as described in the legend to Fig. 1B. Routine manipulations were done by the method of Maniatis et al. (14).

Recombinant vaccinia viruses. Homologous recombination (13) was used to transfer the N gene and associated 7.5-kDa promoter, contained in each of the insertion plasmids pSL125, pSL73, and pAQ330, into the TK gene of vaccinia

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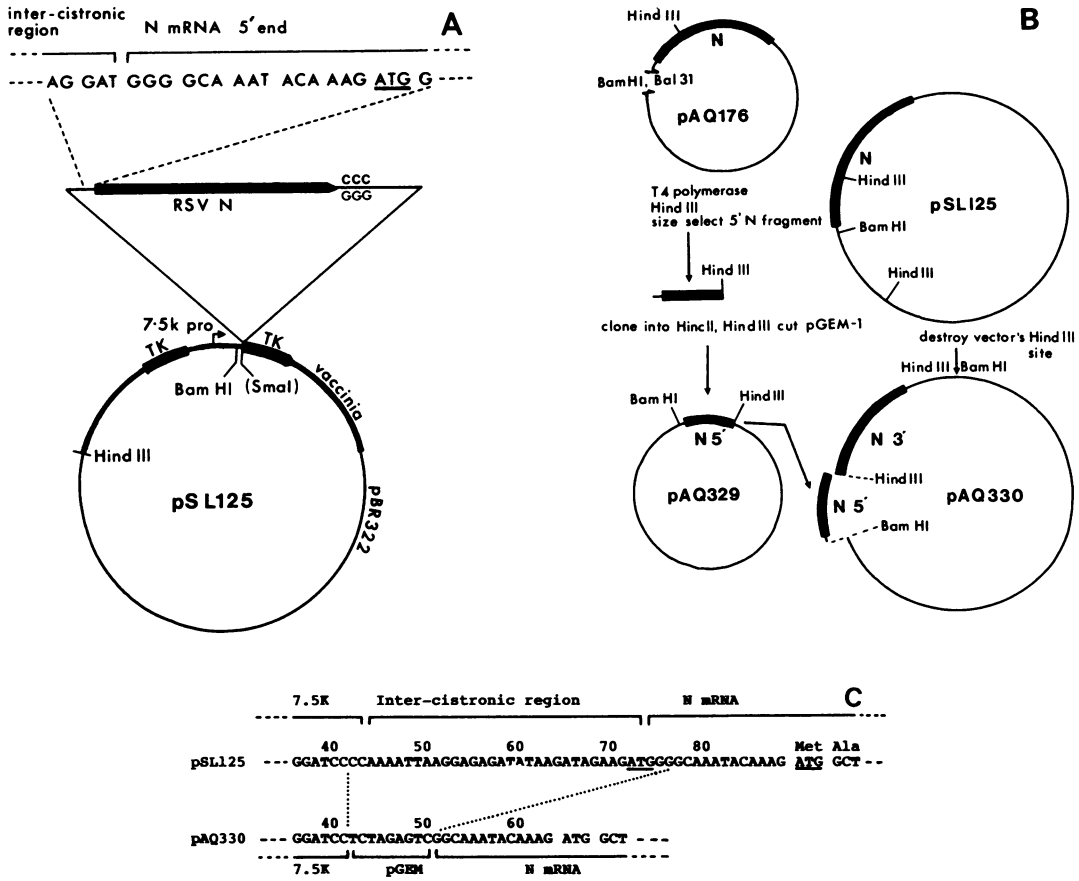

FIG. 1. (A) Structure of vaccinia virus insertion plasmid pSL125. Vaccinia virus sequences flanking the split TK gene are indicated by a line of intermediate thickness; 7.5k pro denotes the 7.5-kDa vaccinia virus promoter (3). (B) Construction of pAQ330, a derivative of pSL125 containing a deletion in the 5'-terminal noncoding region of the N gene. Manipulations were performed on a copy of the N gene that had been inserted into pGEM-1. This plasmid (pAQ176) was opened with *Bam*HI on the 5' side of the N gene. The ends of the DNA were resected with *Bal* 31 exonuclease and made flush with T4 DNA polymerase. Terminal *Hin*dIII fragments were fractionated by agarose gel electrophoresis, and an appropriate size class of fragments was cloned into pGEM-1 cut with *Hin*cII and *Hin*dIII. The *Bam*HI-*Hin*dIII N-gene fragment from one such clone (pAQ329) was substituted for the homologous region of pSL125, to produce the desired vaccinia virus insertion plasmid, pAQ330. To facilitate this final step, the extra *Hin*dIII site in pSL125 was first destroyed by partially digesting the plasmid with *Hin*dIII, filling the ends with T4 DNA polymerase, and recircularizing. (C) Nucleotide sequence of pAQ330 across the site of the deletion: comparison with the sequence of pSL125. The sequence of the *Bam*HI-*Hin*dIII fragment, end labeled at the *Bam*HI site, was determined by the method of Maxam and Gilbert (15).

virus (strain WR), the resulting recombinant viruses being named VVN125, VVN73, and VVN333, respectively. Tk^- recombinants were selected by plaque assay in the presence of 5-bromodeoxyuridine and identified by dot blot hybridization by the procedure of Gillespie and Bresser (8). Viruses were purified by one or two further plaque isolations, and stocks were grown in HEp-2 cells.

Analysis of virus gene expression. Cytoplasmic RNA (27) and protein were prepared from HEp-2 cells 20 to 22 h postinfection with human RS virus (strain A2) or 4 h postinfection with vaccinia virus and its recombinants. In studies of RNA synthesis, late vaccinia virus functions were inhibited by treating the cells with 100 μg of cycloheximide per ml at the time of infection. RNA was recovered by phenol extraction, electrophoresed on 1.5% agarose gels containing 6 M urea at pH 3.5 (29), and blotted onto diazobenzyloxymethyl paper. Blots were hybridized with labeled RNA probes transcribed in vitro from DNA inserted into pGEM-1, using T7 or SP6 RNA polymerase. For protein analyses, cells were lysed at 0°C in 0.5 ml, per 35-mm plate, of 0.01 M Tris hydrochloride (pH 7.4)–66 mM EDTA–1% Nonidet P-40–0.4% sodium deoxycholate, and the nuclei were removed by centrifuging for 3 min in an Eppendorf centrifuge. RS virus-specific proteins were immune precipitated and electrophoresed as described previously (28).

Vaccination and challenge of mice. Groups of five specific-pathogen-free BALB/c mice were vaccinated with vaccinia virus recombinants either intraperitoneally or intranasally under anesthesia. Three weeks later, blood samples were collected from the tail vein and assayed for antibody by enzyme-linked immunosorbent assay using sonicated RS virus-infected HEp-2 cells as antigen and goat anti-mouse immunoglobulin G coupled to horseradish peroxidase (KPL Laboratories, Inc., Gaithersburg, Md.) to detect RS virus-specific mouse immunoglobulin. The mice were then challenged intranasally with 2×10^4 PFU of RS virus; after 5 days, they were killed and their lungs were assayed for virus (24).

RESULTS

Structures of vaccinia virus recombinants. The N gene of RS virus, linked to a copy of the vaccinia virus 7.5-kDa

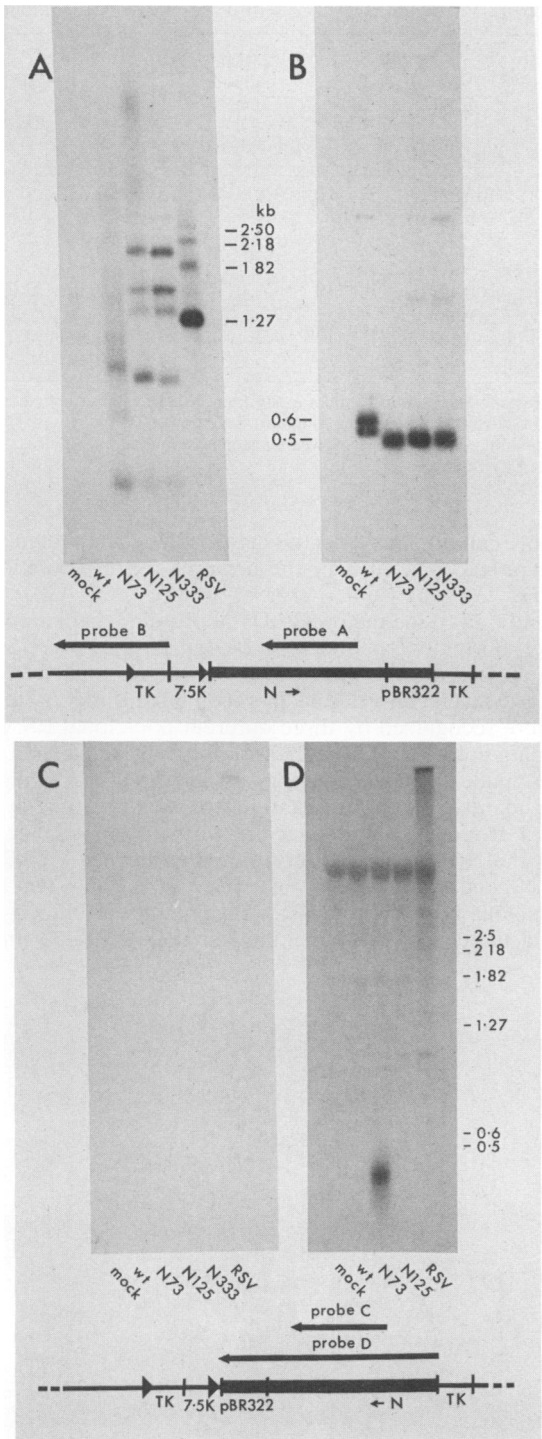

FIG. 2. Positive-sense (A and B) and antisense (C and D) RNAs expressed by the vaccinia virus recombinants. (A, B) HEp-2 cells were infected as indicated, and cytoplasmic RNA was electrophoresed on agarose-urea gels. Duplicate blots were probed with labeled RNA complementary to either (A) an internal region of the N gene, located between the *Hin*dIII and *Rsa*I sites, or (B) vaccinia virus sequences upstream of the 7.5-kDa promoter. The map below the gels shows the locations of the hybridization probes. Note that this map is of the plus-sense recombinant vaccinia viruses VVN125 and VVN333; the DNA insert, represented by the thickened line, was inverted in VVN73, and both insert and 7.5-kDa promoter are missing in wild-type (wt) vaccinia virus. (C, D) The experiment illustrated in (C) is an exact replica of that shown in (A) except that a positive-sense hybridization probe was used. A longer probe (D) of the same polarity revealed a short transcript made by the antisense recombinant VVN73, a map of which is shown at the bottom. Sizes are given for RNA markers on duplicate filters.

promoter, was recombined into vaccinia virus, using insertion plasmids of the type shown in Fig. 1A. This report describes studies of three N-gene recombinants. Recombinant VVN125, derived from PSL125 (Fig. 1A), contained the N gene positively oriented behind the 7.5-kDa promoter. Recombinant VVN73 contained the same DNA insert, but in the opposite orientation. Recombinant VVN333, described below, was identical to VVN125 except for a deletion in the 5′-terminal noncoding region of the N gene. In addition to the complete 1,197-nucleotide N mRNA sequence (4), the N-gene DNA fragment carried by recombinants VVN125 and VVN73 retained 30 base pairs of upstream RS virus sequence derived from the region between the 1B and N genes and 334 base pairs of pBR322 joined to the 3′ end of the N gene by an oligo(dC:dG) linker.

Preliminary studies (not shown) with recombinants VVN125 and VVN73 showed that VVN125 induced the synthesis of a protein which comigrated with N and which was recognized by RS virus antisera. The quantity detected was less than that seen in RS virus-infected cells. One explanation for the low level of expression was suggested by the nucleotide sequence of the 5′-terminal noncoding region of the N gene (Fig. 1A, top). The junction between the intergenic region and the start of the normal N message creates an initiation codon, AUG (ATG in the DNA copy), which is in a different reading frame from the N gene and might be expected to inhibit translation of it. The unwanted ATG sequence was removed by making a small deletion in the 5′ noncoding region of pSL125 (Fig. 1B). The nucleotide sequence across the site of the deletion (Fig. 1C) confirmed that the upstream ATG was eliminated, while the correct initiation sites for transcription and translation were left intact. This version of the N gene was also recombined into vaccinia virus, generating the third recombinant, VVN333.

Analysis of recombinant transcripts. Transcription from VVN125 and VVN333 was analyzed by Northern blot hybridization. Figure 2A, B, C, and D shows blots of cytoplasmic RNA electrophoresed on agarose-urea gels. Hybridization with a probe complementary to N mRNA (Fig. 2A) revealed four species of RNA in RS virus-infected HEp-2 cells, which have been identified previously as the major monocistronic N mRNA (length, 1.27 kb) and three longer readthrough transcripts (5). In cells infected with VVN125 and VVN333, several N transcripts were detected, the four major species being between 0.8 and 2.0 kb long. Although the patterns of RNAs made by these two recombinants were similar, each species of VVN333 RNA migrated slightly faster than its VVN125 counterpart, consistent with the 25-base-pair deletion that had been introduced in VVN333. This observation confirms that all four RNA species contained the initiation site for N-protein synthesis. We can also be confident that none of the major transcripts originated from the upstream TK promoter, since the low level of readthrough from that promoter (Fig. 2B) shows that the 7.5-kDa promoter fragment acts as a strong terminator of early transcription. We therefore conclude that all N-gene transcripts of VVN125 and VVN333 started at the 7.5-kDa promoter and terminated at four alternative locations. Furthermore, three of the four mRNA species were long enough to encode a complete N polypeptide. Curiously, VVN73 also

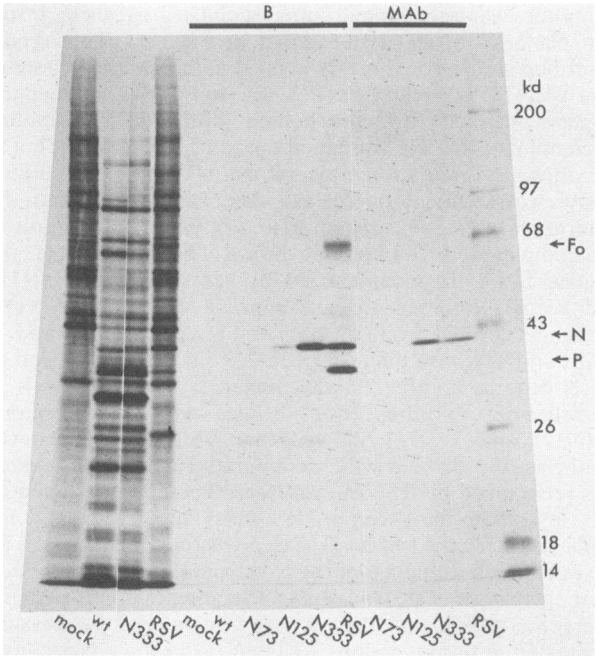

FIG. 3. Synthesis of N protein by vaccinia virus recombinants. HEp-2 cells were infected as indicated and labeled with [^{35}S]methionine for 30 min prior to preparation of cytoplasmic extracts. RS virus proteins were immune precipitated, where indicated, with (B) bovine RS virus antiserum (Burroughs Wellcome) or (MAb) N-specific monoclonal antibody (MAb 15) (21) and analyzed by electrophoresis on 12.5% sodium dodecyl sulfate-polyacrylamide gels. wt, wild type.

synthesized N RNA of positive sense early in infection, despite the inverted orientation of its N gene with respect to the 7.5-kDa promoter. However, these transcripts were shorter than would be expected for a full-length N mRNA (Fig. 2A).

Transcripts of negative sense were sought by using mRNA sense hybridization probes. No RNA complementary to the middle region of the N gene was detected in cells infected with any of the vaccinia virus recombinants (Fig. 2C and D). The band visible at the top of panel C represents minus-strand RS virus genome. However, when the probe extended over the entire 1.6-kb insert (panel D), an RNA species of approximately 0.4 kb was detected specifically in cells infected with the negative-sense construct, VVN73. This shows that the 7.5-kDa promoter did indeed drive transcription of negative-sense RNA in recombinant VVN73, although the product failed to extend as far as the N gene.

Expression of N protein by recombinant vaccinia viruses. Figure 3 shows that the translational activities of the two positively oriented N recombinants, VVN125 and VVN333, were very different from each other. HEp-2 cells infected with VVN333 made large amounts of a 42-kDa protein that comigrated with authentic N. This protein was precipitated specifically by several RS virus antibody preparations, including a polyclonal bovine RS virus serum ("B" in Fig. 3) and an N-specific monoclonal antibody ("MAb"). This protein was also detected in cells infected with VVN125, but at <1/10 the level of VVN333. With the monoclonal antibody, an N-protein band was seen in the VVN125 track after longer exposure than the one illustrated. The 42-kDa band

TABLE 1. Vaccination of mice with vaccinia virus recombinants

Vaccine	Route	Virus dose (PFU)	Mean antibody titer in serum ± SD (log_{10})[a]	Mean RS virus titer in lungs ± SD (log_{10} PFU/g of wet tissue)[b]	Probability of difference[c]
VVN125	Intraperitoneal	2×10^7	3.4 ± 0.2	3.5 ± 0.3	0.068
None			1.5	4.2 ± 0.4	
VVN333	Intraperitoneal	1×10^6	4.1 ± 0.2	3.2 ± 0.3	0.0007
VVN333	Intranasal	1×10^6	4.2 ± 0.3	3.4 ± 0.1	0.0001
None			1.5	4.2 ± 0.1	

[a] Enzyme-linked immunosorbent assay titer at 21 days after vaccination.
[b] At 5 days after challenge (± standard deviation).
[c] Probability of difference between vaccinated and control virus titers, calculated by Student's *t* test.

was not seen in mock-infected cells or in cells infected with wild-type vaccinia virus or the negative-sense recombinant VVN73.

In addition to the major 42-kDa N protein, small amounts of two shorter polypeptides of 24 and 25 kDa were seen. These appeared to be products of the recombinant N gene, since they were only found in recombinant-infected cells, and were recognized by three different polyclonal RS virus sera (data not shown). The 25-kDa polypeptide, seen in Fig. 3, was made in equal amounts by all three recombinants independently of gene orientation and was presumably expressed from a cryptic vaccinia virus promoter located within the N gene. The ability, noted earlier, of VVN73 to make N-gene transcripts of positive sense is consistent with this explanation. By contrast, the amounts of the 24-kDa product varied, VVN125 making less than VVN333 (in the

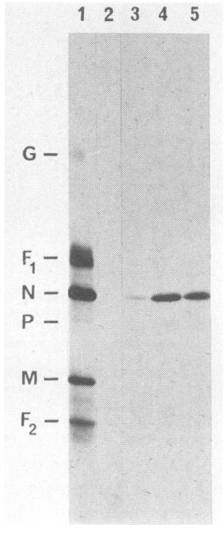

FIG. 4. Immune precipitation of N protein by sera from vaccinated mice. HEp-2 cells were infected with RS virus and labeled with [^{3}H]leucine and [^{3}H]threonine for 16 h before preparation of cytoplasmic extracts. Virus proteins were precipitated with (lane 1) gnotobiotic bovine hyperimmune antiserum, (lane 2) serum from unvaccinated mice, (lane 3) serum from mice vaccinated with recombinant VVN125 intraperitoneally, or (lane 4) serum from mice vaccinated with VVN333 intraperitoneally or (lane 5) intranasally.

former, the band could only be seen after longer exposure than that illustrated) and VVN73 making none at all.

Response of mice to vaccination with recombinants. Previous work with the inbred BALB/c mouse system has shown a protective role for the RS virus G and F proteins when expressed individually from recombinant vaccinia virus vectors (20, 26). We therefore used BALB/c mice to test the efficacy of the N recombinants against live-virus challenge. Mice were immunized with recombinant VVN125 or VVN333 by the intraperitoneal or intranasal route. Three weeks after immunization, mice were bled to assay for serum antibody and the animals were then challenged by intranasal inoculation of 2×10^4 PFU of RS virus. Enzyme-linked immunosorbent assays demonstrated the presence of RS virus-specific antibodies in the sera of mice 21 days after vaccination (Table 1). Higher anti-RS virus titers were present in the sera of animals vaccinated with VVN333 than in those vaccinated with VVN125. The high-expressing recombinant VVN333 generated 5-fold more RS antibody than recombinant VVN125 even though the dose of VVN333 was 20-fold lower. This antiserum was specific for N protein as shown by its ability to specifically immunoprecipitate N protein from lysates of RS virus-infected cells (Fig. 4).

After challenge with RS virus by the intranasal route, mice that had been vaccinated with recombinant VVN333 showed significantly lower titers of RS virus in their lungs compared with unvaccinated controls (Table 1). These results show that expression of high levels of RS virus N protein can provide partial protection of the lower respiratory tract against live-virus challenge.

DISCUSSION

Vaccinia virus provides a uniquely versatile eucaryotic expression vector because of its high immunogenicity, its broad host range, and the nondefective nature of its recombinants. These properties enable one to examine at the molecular level the behavior of individual recombinant viruses in cultured cells and then to study the immune responses to the same recombinants by inoculation of experimental animals. We have used this approach to study both the immunogenicity of several individual RS virus proteins and the response of different branches of the immune system (2, 20, 26).

In the work described above, we successfully expressed the N-protein gene of human RS virus from vaccinia virus recombinants both in infected cells in culture and in inoculated mice. A potential problem in expressing foreign genes from early vaccinia virus promoters is exemplified by the fact that only one of five 7.5-kDa promoter transcripts ended at an authentic vaccinia virus termination site. Thus, only the longest (2.0 kb) N mRNA made by VVN125 and VVN333 (Fig. 2A) extended as far as the 3′ end of the TK gene, the others ending at cryptic termination sites within the inserted DNA. Transcription from the 7.5-kDa promoter in the negative-sense construct, VVN73, also ended prematurely. The termination signal for early vaccinia virus transcription was recently shown to have the consensus sequence TTTTTNT, where N is preferentially A (18). The signal appears once in the N gene, at nucleotide 680 in the mRNA sequence (4), in the form TTTTTGT. The predicted size of a recombinant transcript terminating at this site coincides with the observed size of the smallest N-specific mRNA made by VVN125 and VVN333 (allowing for the fact that termination occurs 50 to 70 nucleotides downstream from the signal). It is also easy to explain how a 3′-terminal poly(A) tract can completely block synthesis of antisense RNA as we have observed before (1), since its complement would be recognized as the reiterated termination signal TTTTTTT.

The availability of two vaccinia virus recombinants differing only in the 5′-terminal noncoding region of the N gene provided an opportunity to test the translational effect of an upstream initiation codon in a vaccinia virus mRNA. This AUG codon lies within a sequence favorable for initiating translation and, according to the ribosome-scanning model (12), would be expected to depress N-protein synthesis strongly. Until now, however, it has not been certain whether the rules of the scanning model apply to vaccinia virus; the fact that late vaccinia virus messages tend to be polycistronic and often lack a favorable upstream consensus sequence at their initiation sites (19) suggests that they may not. Our analyses of RNA and protein expressed by the N genes of VVN125 and VVN333 show that removal of an upstream AUG codon can increase significantly the translational activity of a vaccinia virus mRNA, in accordance with the scanning model.

Vaccination with recombinant VVN333 resulted in significantly reduced RS virus titers in the lungs of mice following live-virus challenge. This protection, however, was not as great as that provided by vaccination with recombinants that expressed the F or G surface glycoprotein (20, 26). It is unlikely that the humoral antibody generated against the nucleoprotein played a major role in this protection since the nucleocapsid protein is an internal viral protein and the N-specific mouse antisera failed to neutralize RS virus in vitro. Moreover, passive transfer of monoclonal antibodies to N protein did not protect mice (23). However, it has been shown recently that infection with recombinant VVN125 confers on autologous target cells the ability to be lysed by human or murine cytotoxic T cells induced by RS virus infection (2). Moreover, murine cytotoxic T cells are induced by recombinants carrying the N gene (R. M. Pemberton, M. J. Cannon, P. J. M. Openshaw, L. A. Ball, G. W. Wertz, and B. A. Askonas, submitted for publication) so it seems likely that this cell-mediated immune response is primarily responsible for the partial protection seen in vaccinated mice.

Cytotoxic T cells have previously been implicated in clearing RS virus infections (7), and this role has recently been confirmed by experiments in which primed T cells were passively transferred into nude athymic, or irradiated, mice persistently infected with RS virus (M. J. Cannon, E. J. Stott, G. Taylor, and B. A. Askonas, unpublished data). The results in this paper show that it is also possible, in principle, to exploit the cellular immune system prophylactically. Although the degree of protection provided by the N-gene recombinant was less than that provided by recombinants carrying the RS virus genes for the G or F glycoprotein (6, 16, 20, 26), there are potential advantages to using an internal viral antigen to stimulate a cellular immune response, one of which is that it would be cross-typic. A double recombinant that expresses both the N protein and a surface glycoprotein of RS virus might elicit the optimum protective response. Such a recombinant has been constructed (L. A. Ball, A. M. Q. King, and G. W. Wertz, manuscript in preparation) and will shortly be tested for its efficacy as a vaccine.

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